IN THE SPECIFICATION:

Please replace the first paragraph on page 1, with the sub heading of 'Scope of the invention" with the following:

FIELD OF INVENTION ~~Scope of the invention~~

The invention relates to the production of interferon alpha 5 for use in compositions useful in the treatment of liver diseases of viral origin.

Please replace the third paragraph on page 1, with the sub heading of 'State of the art" with the following:

BACKGROUND OF INVENTION ~~State of the art~~

Infected cells can recognize the presence of a virus by sending out signals which result in the transcription and secretion of type I interferon (IFNα and IFNβ). IFNα is a family of thirteen polypeptides (subtypes) coded by different genes. IFNβ is a glycoprotein produced by a single gene. Different cell types produce both IFNα and IFNβ (1, 2).

Please add the following new paragraphs after 4th paragraph on page 7 which begins "24. Sarobe".

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1 A-B: Expression of alpha interferon/β-actin RNAm (ordinate) in peripheral

blood mononuclear cells (A) and in the liver (B) of healthy controls and patients with chronic hepatitis C (HCV-RNA+) (abscissa).

Figures 2 A-B: Expression of beta interferon/β-actin RNAm (ordinate) in peripheral blood mononuclear cells (A) and in the liver (B) of healthy controls (C) and patients with chronic hepatitis C (HCV-RNA+) (abscissa).

Figures 3 A-B: Relationship between the initial quantity of total RNA (abscissa) and the strength of the PCR product band obtained by amplifying the RNAm of IFNα (•), IFNβ (▲) and β-actin (◆) (ordinate, as counts x mm^2) in PBMC (A) and liver (B) samples.

Please replace the sub heading before the last paragraph on page 7 which starts "Description of the invention" with the following:

~~**DESCRIPTION OF THE INVENTION**~~ **DETAILED DESCRIPTION**

Patients and controls

The expression of IFNα and IFNβ genes was analysed in samples from liver biopsies from 16 patients with chronic hepatitis C (9 men and 7 women, age range 24 to 71 years). Five of these patients showed cirrhosis. The viral genotype was determined in 14 patients and was lb in 10 patients, la in 2 patients and genotype 3 in 1 patient.

Please replace the second paragraph on page 8, which starts "RNAm levels of" with the following:

~~RNAm~~ mRNA levels of IFNα and IFNβ were also determined in PBMC in 25 patients with chronic hepatitis C (14 men and 11 women, age range 24 to 69 years) (four of these patients had cirrhosis) and in PBMC from 23 healthy controls (10 men and 13 women, age range from 25 to 66 years). The viral genotype for these patients was lb in 22 patients, la in two patients and 3 in 1 patient.

Please replace the paragraph bridging pages 9, 10 and 11, which starts RNAm levels of IFNα" with the following:

~~RNAm~~ mRNA levels of IFNα and IFNβ were determined using a quantitative polymerase chain reaction reverse transcription (RT-PCR) method using a thermocycler (Perkin-Elmer Gene Amp PCR system 2400). Prior to reverse transcription 2 μg of total RNA (from both the liver and PBMC) were treated with 1 unit of deoxyribonuclease (DNAse I amplification grade, Gibco-BRL, Gaithersburg, MD, USA) to eliminate possible contaminating DNA. The presence of traces of DNA was checked by including control reactions without reverse transcription. This step is required because of the absence of introns in IFNα and IFNβ genes (18), which made it impossible for us to distinguish the product of PCR from the RNA or possible contaminating DNA. All the controls performed without reverse transcription were negative, indicating the absence of contaminating DNA. Total RNA was transcribed (60 minutes at 37°C) with 400 units of M-MuLV reverse transcriptase (Gibco- BRL, Gaithersburg, MD, USA) in a final volume of 40 μl of 5 x saline solution (250 mM

Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), supplemented with 5 mM DTT, 0.5 mM triphosphate dioxyribonucleotides (Boehringer Mannheim, Mannheim, Germany), 48 units of RNAsas inhibitor (Promega Corporation, MD, US) and 400 ng of random hexamers (Boehringer Mannheim, Mannheim, Germany). After denaturing the reverse transcriptase (95°C, 1 minute) and rapidly cooling over ice, a 10 µl aliquot (0.5 µg) of the cDNA was used to amplify the IFNα and IFNβ by PCR in 50 µl of 10 x PCR buffer (160 mM $(NH_4)SO_4$, 670 mM Tris-HCl pH 8.8, 0.1% Tween 20) supplemented with the direction and antidirection primers (40 ng of each one for IFNα and 60 ng for IFNβ), 1.2 mM $MgCl_2$ and 2 units of Biotaq™ DNA polymerase (Bioline, London, LTK). Control reactions without RNA were performed in all the experiments. As an internal control for each sample a fragment of β-actin cDNA was amplified using a 10 µl aliquot of the cDNA obtained previously. The IFNα was amplified by performing 30 or 33 cycles (PBMC or liver respectively) (94°C, 60°C and 72°C during 20, 15 and 30 seconds for each step respectively), the INFβ was amplified by performing 30 or 35 cycles (PBMC or liver respectively) (94°C, 58°C and 72°C for 20, 15 and 30 seconds for each step respectively) and β-actin was amplified by reacting 18 or 25 cycles (PBMC or liver respectively) (94°C, 55°C and 72°C for 20, 15 and 30 seconds for each step respectively), protocols which avoid interference with the PCR reaction saturation stage. The oligonucleotides (5'-3') d(TCCATGAGATGATCCAGCAG) (SEQ ID NO:2) and d(ATTTCTGCTCTGACAACCTCCC) (SEQ ID NO:3) were used as direction and antidirection primers respectively to amplify a fragment of 274 pairs of bases located between nucleotides 240-514 in the human IFNα gene (19). These oligonucleotides

are direction primers designed to amplify all the subtypes of IFNα. The oligonucleotides D(TCTAGCACTGGCTGGAATGAG) (SEQ ID NO:4) and d(GTTTCGGAGGTAACCTGTAAG) (SEQ ID NO:5) were the primers used to amplify a fragment of 276 base pairs located between nucleotides 349-625 of cDNA of human IFNβ (20) d(TCTACAATGAGCTGCGTGTG) (SEQ ID NO:6) and d(GGTGAGGATCTTCATGAGGT) (SEQ ID NO:7) were the primers used to amplify a fragment of 314 base pairs (nucleotides 1319-2079) of the β-actin gene (21).

Please replace the paragraph bridging pages 11 and 12, which starts with "After the amplification" with the following:

After the amplification reactions 20 μl of the PCR product were run in a 2% agarose gel containing ethidium bromide. The bands obtained were displayed using an ultraviolet lamp and were analysed using a commercial programme (Molecular Analyst/PC, Bio-Rad) capable of digitizing and analysing the image obtained. Finally the values corresponding to the expression of the IFNα and IFNβ genes were standardized with their β-actin correlates. The results are expressed as the quotient between the value of IFNα and IFNβ and the β-actin correlate. Previously we demonstrated that the ~~RNAm~~ mRNA of β-actin was expressed constantly both in the liver and in the PBMC of patients with chronic hepatitis C (22), which has enabled us to standardize IFNα and IFNβ values with those obtained for β-actin.

Please replace the paragraph bridging pages 16 and 17, which starts with "After

extraction of" with the following:

IFNα subtypes in normal liver tissue and PBMC in healthy individuals

After extraction of the total RNA of the normal liver tissue samples the ~~RNAm~~ mRNA of the IFNα was amplified using universal primers for all the IFNα subtypes. The PCR amplification products were then cloned and sequenced. 41 clones from 4 different normal livers were analysed and we observed that the IFNα sequence in the 41 clones was the same and corresponded to the IFNα5 subtype (Table l). These results show that IFNα5 is the only IFNα subtype expressed in normal liver. The partial cDNA sequence of the IFNα5 obtained from all the clones was shown to be SEQ ID NO: 1.

Please replace the second paragraph on page 17, which starts "To compare the" with the following:

To compare the profile of the IFN subtypes expressed in the liver with that expressed in PBMC the total RNA of the PBMC from 5 healthy controls was extracted and the IFNα ~~RNAm~~ mRNA was amplified with the universal primers for all the IFNα subtypes. Of the 43 clones analysed, 15 corresponded to the IFNα5 subtype, 14 to the IFNαl/13, 6 to the IFNα21 and 8 clones to other IFNα subtypes (Table l). These results indicate that the IFNα subtype profile expressed in PBMC differs from that expressed in normal liver.

Please replace the paragraph bridging pages 17and 18, which starts with "The above results" with the following:

IFNα subtypes in liver tissue and PBMC from patients with chronic hepatitis C

The above results show that the normal liver expresses IFNα5, while PBMC express a variety of IFNα subtypes. In the liver parenchyma of patients with chronic hepatitis C there is mononuclear cell infiltrate, an important source of IFNα. This suggests that the profile of IFNα subtypes expressed by the liver in patients with chronic hepatitis C might differ from the profile found in normal liver. To investigate the expression of IFNα subtypes in chronic hepatitis C we extracted the total RNA from liver samples from 3 different patients and 2 PBMC samples. After amplifying the IFNα ~~RNAm~~ mRNA with universal primers for all subtypes, we cloned and sequenced 24 clones of liver tissue and 18 clones of PBMC. As shown in Table 1, the PBMC from patients with chronic hepatitis C expressed IFNα21, IFNα5 and IFNα7 (5, 12, and 1 clones respectively). In the liver tissue from these patients we found subtypes IFNα21, IFNα17 and IFNα1/13 (8, 1 and 2 clones respectively) in addition to the IFNα5 subtype (Table l).

Please replace the second paragraph with sub heading on page 18, which starts "Levels of " with the following:

Levels of expression of IFNα ~~RNAm~~ mRNA in PBMC and the liver of patients with chronic hepatitis C and controls

Total RNA was extracted from PBMC and liver samples from patients with chronic hepatitis C (n=25 and 16, respectively), PBMC samples from healthy controls (n=20) and normal liver tissue samples obtained by laparotomy (n=12). The ~~RNAm~~ mRNA levels of IFNα were determined using the semiquantitative reverse transcription-

polymerase chain reaction (RT-PCR) technique using universal primers to amplify all the IFNα subtypes. The values are expressed as the ratio of IFNα ~~RNAm~~ mRNA to β-actin ~~RNAm~~ mRNA.

Please replace the paragraph bridging pages 18 and 19 which start "We found" with the following:

We found that the levels of expression of IFNα in the PMBC of patients with chronic hepatitis C were significantly increased in comparison with those found in healthy controls (3.2 ± 0.48 against 1.14 ± 0.26; p=0.001) (Figure 1A). This result was expected in a viral infection such as hepatitis C in which the PBMC are infected (14). On the other hand the levels of expression of IFNα ~~RNAm~~ mRNA were significantly reduced in the liver tissue from patients with chronic hepatitis C in comparison with that expressed in normal liver (0.12 ± 0.03 against 0.43 ± 0.12; p=0.003) (Figure 1B).

Please replace the second paragraph on page 19, which starts "As observed" with the following:

As observed previously, IFNα5 is the only IFNα subtype detected in normal liver, while a mixture of subtypes is observed in the liver tissue of patients with chronic hepatitis C. Our findings indicate that in infection by HCV there is a marked reduction in the expression of the IFNα subtype normally expressed in liver tissue. Interestingly, IFNα ~~RNAm~~ mRNA levels in the livers of patients with chronic hepatitis C show a direct correlation with the Knodell index ($r=0.54$; $p<0.05$). This finding, together with the observation that the IFNα subtypes detected in the livers of

patients with chronic hepatitis C are those observed in PBMC suggests that most of the IFNα ~~RNAm~~ mRNA found in the liver in hepatitis C comes from the inflammatory infiltrate. It appears possible that the reduction in the expression of liver IFNα (IFNα5) may play a part in making the HCV infection chronic. As a result, these observations may have therapeutic implications if we also bear in mind the marked antiviral and antiproliferative activity of the IFNα5 described by other authors (9).

Please replace the paragraph and sub heading bridging pages 19 and 20, which starts with "IFNβ, the second" with the following:

Levels of expression of IFN ~~RNAm~~ mRNA in the PBMC and liver of patients with chronic hepatitis C and controls

IFNβ, the second majority form of type 1 interferon, is a glycoprotein produced by a single gene. In viral infections transcription of the IFNα and IFNβ genes is activated or repressed by various mechanisms (15). To analyse the expression of IFNβ in chronic hepatitis C we determined IFNβ ~~RNAm~~ mRNA levels in the same samples of liver tissue and PBMC previously used to determine the expression of IFNα .

Please replace the first paragraph on page 20, which starts "As shown" with the following:

As shown in Figure 2, we observed that IFNβ ~~RNAm~~ mRNA levels (expressed as a ratio against β-actin) were significantly higher in both PBMC and the liver in patients with chronic hepatitis C in comparison with the PBMC findings in healthy controls

and normal livers (1.66 ± 0.2 against 0.88 ± 0.16; p=0.008 in PBMC and 1.37 ± 0.23 against 0.97 ± 0.16; p=0.011 in liver). These results show that while HCV causes IFNα to be repressed in the liver, the expression of IFNβ is increased in both the liver and PBMC. This indicates that VHC modulates the different type I IFN genes in the liver in a different way, and blocks the production of IFNα to permit the overexpression of IFNβ.

Please replace the paragraph and subheading bridging pages 20 and 21, which starts "In order to" with the following:

Relationship between the expression of IFNα and IFNβ genes with viral load, genotype and liver damage in chronic hepatitis C

In order to determine whether the expression of the IFNα or IFNβ genes can be related to viral load or genotype we quantified the C virus RNA in the serum of all patients using the competitive PCR technique and determined the VHC genotype using a hybridization method with specific test materials. We found no correlation between the expression of the IFNα or IFNβ genes (in the liver or PBMC) and C virus RNA levels in serum or the viral genotype. Analysing the relationship between the expression of the type I IFN genes and the severity of liver damage in patients with chronic hepatitis C we found that IFNβ ~~RNAM~~ mRNA levels in the liver correlated directly with serum aspartate aminotransferase values (r=0.64, p=0.008) and the Knodell index (r=0.66, p=0.006). Likewise the IFNα ~~RNAm~~ mRNA values in the liver showed a direct positive correlation with the Knodell index as mentioned previously.

Please delete page 23 with the sub heading of "Description of the Figures".

IN THE DRAWINGS:

Replacement sheets of the drawings are submitted herewith to correct the informalities noted on Form PTO 948 attached to paper no. 15. Annotated sheets showing the proposed changes are also submitted herewith.